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– Utilitiy Patent Specification –

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Invention:

Reagents for Protection of Peptide/Protein Carbamylation in Urea Solutions Utilizing Non-Ethylene-diamine Like Compounds

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Title of the Invention

Reagents for Protection of Peptide/proteins Carbamylation in Urea Solutions Utilizing Non-Ethylene-diamine Like Compounds

Related Applications

This application claims priority to provisional application no. 60/449,091, filed on February 21, 2003 and titled "Reagents for Protection of Peptide/proteins Carbamylation in Urea Solutions Utilizing Non-Ethylene-diamine Like Compounds."

Field of the Invention

Embodiments of the present invention generally relate to a class of molecules that substantially prevent the carbamylation of peptides.

Background of the Invention

It has been recognized for many years that cyanate readily reacts with certain amino acid side chain functional groups of a peptide [see G. R. Stark, Method In Enzymology 11, 590-594 (1967)].

Urea-containing solutions are commonly used to solubilize proteins. One of the disadvantages of the use of urea is that it can dissociate into cyanic acid. The cyanate thus formed often reacts with the primary amine in the protein to yield a carbamylated derivative. This derivative may have biological and antigenic properties that are different from those of the native protein. As a result that the therapeutic efficacy of a carbamylated protein may be compromised. In addition, irreversible carbamylation of primary amines on proteins or peptides could complicate the purification process and/or reduce the biological activities of therapeutic bioproducts.

At equilibrium, an 8 M urea solution may contain 0.02 M cyanate, according to the following reaction:

$$H_2N\text{-}CO\text{-}NH_2 \Leftrightarrow CNO^- + H^+ + NH_3^-$$
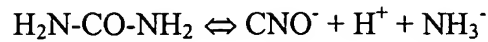
The nonspecific, pH dependent binding reaction between cyanic acid and protein is called carbamylation:

$$CNO^- + \text{amino acid side chains} \Rightarrow \text{carbamylated protein.}$$
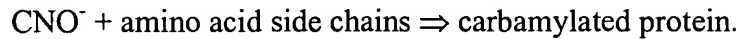
The cyanate reaction with -NH$_2$ side chains is irreversible and has an appreciable rate. Also, slightly acidic conditions promote a rapid reaction of cysteine sulfhydryls with residual cyanate derived from urea. However, the carbamylation of the –SH and –imidazole side chains is reversible in slightly alkaline pH. The carbamylated residues are neutral, so that the proteins become less charged and can be reflected in their ion exchange chromatographic behavior.

Protein carbamylation is a major issue both *in vivo* and *in vitro*. Lippincott and Apostol (1999) have shown that hemoglobins had a significant level of carbamylated cysteines as an artifact of protein digestion in the presence of urea. Hasuike et al. (2002) have recently shown that cyanate can induce hemolysis by carbamylation of erythrocytes. Thus, carbamylated hemoglobin serves as a marker of posttranslational protein modification associated with such uremic complications as atherosclerosis. Oimomi et al. (1987) measured the activity of carbamylated insulin and showed that both immunological and biological activities changed. In addition, Crompton et al. (1985) had shown that the carbamylation of lens proteins by cyanate causes conformational changes that lead to cataracts. *In vitro*, the carbamylation of proteins results in lower protein

solubility and biological activity, that can lead to a low purification yield and a difficult purification process.

Different methods to prevent carbamylation have been proposed. Lowering the temperature slows down both the cyanate formation and subsequent carbamylation, but increases the viscosity that can impact downstream processes such as filtration and chromatography. Deionization of urea solution only temporarily removes the cyanate from solution. Lowering the pH to 2 decreases cyanate formation but is unattractive for most proteins. Amine-specific derivatization and deprotection is not a convenient quantitative approach. While these approaches have applications in special circumstance, none can be generally applied in the field.

Since cyanate formation in the urea buffer cannot be prevented under the condition of normal protein purification, an alternative approach would be to remove cyanate as it forms. A search for CNO^- scavengers has been reported (*e.g.* $H_2N\text{-}CH_2\text{-}CH_2\text{-}NH_2$ for insulin, see DiMarchi patent, 1986). A good protection agent is considered to be inexpensive, inert, soluble, and readily removable. It has to provide high level of protection, and possibly form irreversible complexes with HOCN at neutral pH. Scavenger design is difficult because each functional group has different reactivity, and the protection mechanism is not clear. Good candidates could be amines that are more reactive with CNO^- than the primary amine groups of proteins, and have more than one functional group that are sterically unhindered for faster kinetics.

Two alternatives which can be considered for diminishing losses due to carbamylation are addition of a protective scavenger and reversible amine protection. While the latter approach may permit complete protection it is not always desirable due

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primarily to the difficulty in achieving quantitative amine-specific derivatization and deprotection. If an appropriate reaction scavenger is available, its utilization is a more desirable and less expensive and demanding alternative to reversible protection. An ideal scavenger, of course, is one that is inexpensive and that provides complete protection from modification while otherwise being totally inert to all other reaction components. Since proteins contain a wide range and diversity of functional groups, each of which possesses a different reactivity toward a particular reagent, it is difficult a priori to predict an effective scavenger. While carbamylation is most rapid at sulfhydryl and imidazole sites, the resulting reaction products are of little concern due to their rapid reversal in slightly alkaline buffers. Modification of peptidyl primary amines (for example, NH$_2$ - terminus and lysine residues) occurs at an appreciable rate and, for all practical purposes, is irreversible [see G. R. Stark, W. H. Stein, and S. Moore, **J. Biol. Chem.** 235, 3177- 3181 (1960)]. At each site of primary amine carbamylation the peptide is reduced in physiological buffers one positive charge, thereby often resulting in diminished peptidyl solubility and/or biological activity. Since cyanate is an equilibrium product of aqueous urea solutions [see J. R. Marier and D. Rose, **Anal. Biochem.** 7, 304-314 (1964)], all peptides containing reactive functional groups, when handled in the presence of urea, are susceptible to irreversible carbamylation. Urea, being an excellent peptidyl solvent due to its ability to disaggregate structural order, facilitates carbamylation. These undesirable derivatized forms not only represent immediate losses in yield but also constitute complications in purification processes.

To diminish carbamylation of peptides in many prior art urea solutions, the solutions were freed of cyanate prior about immediately prior to use, and all chemical manipulations were conducted at reduced temperatures.

There are several prior art methods and/or processes for the removal of cyanate from mediums and/or solutions. One process, the removal of cyanate by deionization, or through pH reduction to below 2.0, is at best temporary, since ammonium cyanate reappears as an equilibrium product of aqueous urea. As well, the low temperature operational restriction results in slower chemical reactions and complicated operations.

The DiMarchi process, and especially the '513 patent, teaches that if an effective scavenger is to be found, it is important first to determine the optimum conditions for carbamylation. In fact, it was the DiMarchi process that aided in the understanding that there are more than one reactive species when considering the carbamylation of proteins during synthesis. It is commonly understood in the art that if cyanate is the reactive species, the rate of reaction should increase with increasing pH until a limit is reached at a pH slightly above the pKa of the amine. However, if cyanic acid is the reactive entity, the relative rate of reaction with an amine should be biphasic with a pH optimum of approximately 6.5. DiMarchi further taught that an ideal reagent for use in preventing peptide carbamylation (scavenge) during synthesis would have the characteristics of being inert to peptides, capable of forming irreversible complexes with cyanic acid at approximately pH 6.5.±2.0, and be a 1,2-ethylene diamine-like compounds or a compound structurally related to 1,2-ethylene diamine and having some carbamylation inhibition and/or reduction characteristics similar thereto. The DiMarchi process defines a compound structurally related to 1,2-ethylene diamine like compound as:

$$R_3R_4N-\underset{\underset{R_1}{|}}{CH}-\underset{\underset{R_2}{|}}{CH}-NH_2$$
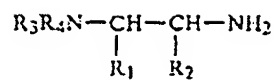
In which R_1, R_2, R_3, and R_4 are groups which, as a composite, do not exert significant changes (1) in the pKa values and (2) in the steric accessibility of the respective amino acid groups relative to the properties of 1,2-ethylene diamine itself. DiMarchi stresses that it is the steric arrangement of the 1,2-ethylene diamine like compound that provide for the scavenging ability.

However, while the 1,2-ethylene diamine like compounds possess good cyanate scavenging ability, they are highly basic and strongly influence pH and buffering capacity when used at the concentration suggested by DiMarchi. Therefore the artfield is in search of other compounds and/or groups of compounds that function as carbamylation inhibitors without this disadvantage of the 1,2-ethylene diamine like compounds. Such compounds should either be much more effective scavengers than 1,2-etylene diamine, so that they can be used in sub-milimolar concentration, or significantly less basic than 1,2-ethylene diamine, preferably with low or no net charge at the experimental conditions, having low impact on the buffering capacity of typical biological buffers when used at a milimolar concentrations. Alternatively, such compounds would have a buffering capacity within or close to the neutral range and could be used as buffers.

SUMMARY OF THE INVENTION

Embodiments of the present invention generally relate to a class of non-ethylene diamine like compounds that are capable of substantially inhibiting and/or delaying carbamylation of peptides. In particular, various embodiments of the present invention

relate to compounds that provide protection to proteins from carbamylation in the presence of cyanate and/or in a urea buffer.

Using bovine pancreatic ribonuclease (RNase A) as a model protein, it has been found that several non-ethylene diamine like compounds, such as glycinamide, histidine, 4-hydroxyl proline and some dipeptides, such as Glycine-Glycine (Gly-Gly), and Glycine-Histidine (Gly-His), significantly inhibited carbamylation of RNase A. Unexpectedly, these compounds are not 1,2-ethylene diamine like compounds and are not expected to act as carbamylation inhibitors as defined in the DiMarchi process.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "ethylene diamine like compounds" means and refers to a compound structurally related to, or like, 1,2-ethylene diamine and having some carbamylation inhibition and/or reduction characteristics similar thereto.

The processing of peptides as contemplated herein encompasses any of a wide range of peptide processing. Typical, non-limiting, examples are purification, chemical modification, including, e.g., peptide sulfitolysis, and other such peptide processing steps.

Accordingly, in an embodiment, the present invention comprises a process for inhibiting and/or delaying carbamylation of a peptide/protein in a urea and/or cyanate containing solution during processing of said peptide/protein comprising the step of

adding a carbamylation inhibiting compound to the process wherein said compound is not an ethylene diamine like compound.

In an embodiment, the compound is selected from the group consisting of glycinamide, histidine, 4-hydroxyl proline, Glycine-Glycine (Gly-Gly), and Glycine-Histidine (Gly-His).

Generally, the concentration of the scavenger compound used in the process of this invention is within the range from about 1 mM to about 150 mM. In an embodiment, the concentration of the compound is within the range from about 10 mM to about 100 mM, based upon the total processing medium. In another embodiment, the concentration of the compound is within the range from about 25 mM to about 50 mM. However, the concentration of the compound may vary according to the concentration of the cyanate in solution.
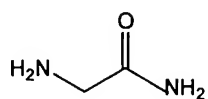
Carbamylation inhibition during processing is available for essentially any peptide and/or protein, irrespective of structure, when subjected to conditions in which amounts of cyanic acid can be expected to be present. Thus, for example, and not by way of limitation, peptides/proteins such as ribonucleases, insulin A-chain, insulin B-chain, proinsulin, C-peptide, pancreatic polypeptide, growth hormone, growth hormone releasing factor, insulin-like growth factor, somatostatin, and, others are suitable for use with the novel non-ethylene diamine like compounds of the present invention. Preferred peptides/proteins are soluble in urea and readily carbamylate in the presence of urea.

The compounds described in this invention do not possess the diamine functionality characteristic of ethylene diamine-like compounds described by DiMarchi, and exhibit much lower net charge at neutral pH making them superior to ethylene-diamine-like compounds as buffer additives. In an embodiment, the compound is selected from the group consisting of glycinamide, histidine, 4-hydroxyl proline, Glycyl-Glycine (Gly-Gly), and Glycine-Histidine (Gly-His). Surprisingly, the compounds selected from this group show comparable cyanate-scavenging and carbamylation-protecting properties to ethylene diamine while lacking the diamine functionality. Unexpectedly, it has been further observed, that some of the cyanate scavenging compounds described in the current invention do not possess any primary amine or sulfhydryl functionality while still showing the ability to scavenge cyanate and protect against carbamylation, the examples being diethanolamine and hydroxyproline.
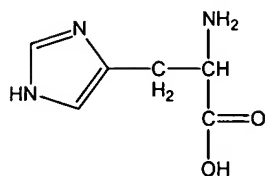
Further unexpectedly, it has been found that it is not necessary or required that the compound by sterically unhindered as proposed by DiMarchi for the compound to function as a cyante scavenger. Compounds selected from a group of non-ethylene diamine like compounds that varied sterical constrains around the amino group inhibit and/or delay carbamylation of peptides/proteins with comparable results. In an embodiment, the compound is selected from the group consisting of glycinamide, histidine, 4-hydroxyl proline, Gycine-Glycine (Gly-Gly), and Glycine-Histidine (Gly-His).

The structures of the compounds are as follows:
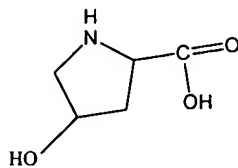
Glycinamide
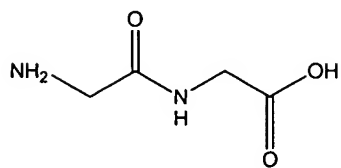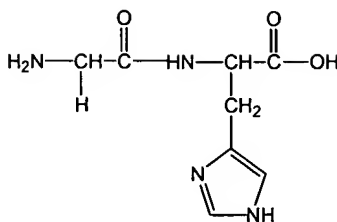
$$H_2N-CH_2-C(=O)-NH_2$$

Histidine

(imidazole ring)—$C(H_2)$—$CH(NH_2)$—$C(=O)OH$

4-hydroxyl proline

(pyrrolidine ring with N-H, C=O, OH, and HO substituent)

Gycine-Glycine

$$NH_2-CH_2-C(=O)-N(H)-CH_2-C(=O)OH$$

Glycine-Histidine (Gly-His)

$$H_2N-CH-C(=O)-HN-CH-C(=O)-OH$$

(with side chains: CH below the first carbon marked H, and CH_2 below the second carbon leading to an imidazole ring N—NH)

As can be seen, when compared to ethylene diamine like compounds,

$$R_3R_4N-CH(R_1)-CH(R_2)-NH_2$$
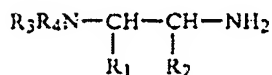
, selected compounds of the present invention, such as 4-hydroxyproline or diethanolamine containing a secondary amine group, cannot be referred to as sterically unhindered. Therefore, it is proposed that the effectiveness of the compounds of the present invention is determined by the stability of a cyanate-scavenger adduct rather than any sterical constrains within the scavenger itself. Such compounds include but are not limited to 4-hydroxyproline, histidine, histidine-glycine, and diethanolamine, consequently, the effectiveness of the compounds described in the present invention can not be predicted from the worked described by DiMarchi.

In another surprising fact, the pKa value(s) of the amino group of a compound may vary significantly from the pKa of an ethylene diamine like compounds while still retaining a good ability to inhibit and/or delay the carbamylation of peptides/protein during processing. DiMarchi proposed that the low pK of approximately 7.5 of one of the amino groups of ethylene diamine or ethylene diamine-like compounds was essential for good scavenger properties. In various embodiments of the present inventionan amine may be used with a pKa of about 8.20. Notably, such a mono-amine compounds would not be predicted to function as carbamylation inhibitors and/or delayors, a suitable

example being glycineamide and/or glycine-glycine. In another embodiment, three groups having varying pKa values of about 1.82, about 6.04 and about 9.33, a suitable example being Histidine with a –COOH, a NH.sub.2, and a side chain. In another embodiment, two groups with pKa values of about 1.92 and about 9.73, a suitable example being hydroxy-proline.

As defined above, embodiments of the present invention encompass numerous processes to which peptides/proteins are subjected. In an embodiment, the process is solubilizing the peptide/protein in urea. In another embodiment, the process is purification of peptide/protein. However, the invention may comprise other processes.

Various embodiments of the present invention inhibit carbamylation of the peptide/protein to varying degrees. In an embodiment, the carbamylation percent protection is about 100% after three weeks. In another embodiment, the compound inhibits carbamylation of ribonuclease A to a greater extent than does 1,2-ethylene diamine inhibit the carbamylation of ribonuclease A. Preferred compounds for comparison comprise a compound selected from the group consisting of histidine, 4-hydroxyl praline, and Gly-Gly.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and the appended. Claims are intended to cover any variations, uses, or adaptations of the invention

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following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth whether now existing or after arising. Further, while embodiments of the invention have been described with specific dimensional characteristics and/or measurements, it will be understood that the embodiments are capable of different dimensional characteristics and/or measurements without departing from the principles of the invention and the appended Claims are intended to cover such differences. Furthermore, all patents and other publications mentioned herein are herby incorporated by reference.

Examples:

In this experiment, a series of amines, amides, amino acids, and di- and tri-peptides were tested and compared with 1,2 ethylene diamine, and their efficiency quantified as % protection against RNase A carbamylation. In our study on cyanate scavengers for protein carbamylation protection, bovine pancreas RNase A (124 amino acid residues, ~14 kDa) served as the model protein. It is a notably stable enzyme that may be inactivated by long exposure at moderate temperature to urea (CNO^-) by chemical changes at the 8 Cys, 10 Lys, 4 Arg, 4 His residues.

Materials and Methods

Cyanate Carbamylation Study

A 2 mg/ml stock solution of bovine RNase A (Sigma), a 100 mM stock solution of sodium cyanate (Sigma), and a stock solution of 250 mM sodium phosphate pH 7.9 were prepared in water and stored at −70°C. The tested scavengers (Table 1) were prepared in 0.5 M stock concentrations, adjusted to pH 8 using HCl or NaOH, and stored at room temperature. The pH of the histidine solution was not adjusted (it could not be adjusted properly). Due to solubility limitation, a 0.2 M stock solution was made for GlyGlyGly, tryptophan, melamine, triaminopyrimidine, and indole. All amino acids were the L isomer.

RNase A, at 1 mg/ml, was carbamylated by incubation with cyanate at room temperature. Different scavenger concentrations (100, 50, 10 and 5 mM) were tested for protective potential. The final concentrations of other components in the carbamylation reaction mixture were 5 mM for CNO⁻ and 50 mM sodium phosphate pH 7.9.

The controls were added to each reaction setup: A negative control, which had neither cyanate nor scavenger, was used for quantifying the RNase natural decay. A positive control, which had 5 mM cyanate, but no any scavenger reagent, was used for estimating the completion of the carbamylation reaction.

In all experiments, 5 mM cyanate was added last.

Non-carbamylated RNase A was quantitatively determined by HPLC on a Mono

S column (Amersham Biosciences, Piscateway, NJ). Aliquots of the carbamylation

reaction mixture were taken at 0, 3, 7, 14, 21 day time points, and the samples were

analyzed by HPLC. If not used immediately, the samples were frozen at –70°C. Prior to

HPLC, the samples were titrated to pH 5 using 38% acetic acid solution to a final 1:1

dilution. A buffer system consisting of 50 mM ammonium acetate pH 5 (buffer A) and 1

M ammonium acetate pH 5 (buffer B) was used for separation of carbamylated and non-

carbamylated RNase species. A gradient was used from 10% B to 70% B over 14 min.

Then the column was washed with 70% buffer B for 2 min. At 16 min the mobile phase

was switched back to 10% B to equilibrate the system before next injection. All

chromatographic separations were carried out at 10 °C, using a mobile phase flow rate of

1 ml/min, and 100 μl sample injection volume.

The amount of remaining non-carbamylated RNase A in the test was converted to percent

carbamylation protection. To account for differences in samples, time 0 of each tested

group was considered to be 100% protection based on the assumption that there is no

carbamylation at time point 0. The data were further corrected point-by-point for natural

protein decay.

Cyanate Assay

To check for CNO-/scavenger interaction and estimate reaction kinetics, the same

reaction setup was used, but no protein was added to final mixture. Aliquots were taken

at 2h and 24h time points and treated as above. A modified HPLC procedure (Black &
Schulz, 1999) was used for free cyanate detection. The HPLC samples were diluted 1:20
with water. Separations were carried out at room temperature, using a mobile phase flow
rate of 1.2 ml/min and 100 μl injection volume. The results were normalized to 5 mM,
the starting cyanate concentration. In some cases, the scavenger peak overlaps the
cyanate peak, so the integration values are smaller than expected.

Urea Carbamylation Assay

A 9 M urea (JT Baker) stock solution was made fresh in 50 mM sodium
phosphate, pH 7.9, and used immediately to make up a 1.1 mg/ml RNase A stock
solution. The final urea concentration in the carbamylation mixture was 8.1 M and the
RNase A was 1 mg/ml. No sodium cyanate added to the reaction mixture. The
experiment further proceeded as described in cyanate carbamylation study.

Results and Discussion

Cyanate Carbamylation Study

Table 1 summarizes all compounds tested for their potential protection against
RNase A carbamylation by cyanate. The percent protection of RNase A was determined
for 100 mM tested reagents after three weeks in the presence of 5 mM cyanate. The 5
best possible protection reagents, which showed a higher than 90% protection level, were

glycinamide, L-Histidine, hydroxyl-Proline, glycylglycine and glycylhistidine. The time

and concentration dependence of these compounds on RNase A carbamylation is shown

in Table 2. In the table, the positive control was set with RNase A, 5 mM cyanate, but

without any potential protection reagents. The negative control was only the RNase A in

test buffer. Data from Table 2 clearly proved that the carbamylation of RNase A by

cyanate was inhibited with 50 or 100 mM tested reagents, compared to the positive

control.

Table 1. Tested compounds in the carbamylation experiment. The percent protection was determined for 100 mM scavenger after 3 weeks in the presence of 5 mM cyanate.

Tested Compound	Carbamylation protection (%)
1,2-Ethylene diamine	99
Ethanolamine	< 70
Diethanolamine	82
Taurine	< 70
L-Glycine	77
Glycinamide	93
L-Lysine	77
L-Arginine	77
Putresceine (Tetramethylenediamine)	< 70
L-Histidine	100
Histamine (2-(4-imidazolyl)-ethylamine)	< 70
Imidazole	< 70
L-Trptophan	< 70
Indole	< 70
L-Proline	< 70
HO-Proline (trans-4-hydroxy-L-proline)	100
Gly-Gly	100
Gly-His	95
His-Gly	85
Gly-Gly-Gly	77
Melamine (2,4,6-triamino-1,3,5 triazide)	< 70

2,4,6-triamino pyrimidine	< 70

Table 2. Selected results of RNase cyanate carbamylation study.

Tested compounds and concentration		Carbamylation Protection (%)				
		Day 0	Day 3	1 week	2 week	3 week
1,2-Ethylene diamine	100 mM	100	98	96	96	93
	50 mM	100	94	93	93	90
L-Histidine (His)	100 mM	100	93	97	102	102
	50 mM	100	68	70	76	77
Glycinamide (GLA)	100 mM	100	97	96	94	93
	50 mM	100	93	92	92	87
Glycylglycine (GlyGly)	100 mM	100	98	98	100	101
	50 mM	100	91	92	94	93
Gly-His (GlyHis)	100 mM	100	97	95	97	95
	50 mM	100	95	95	96	91
Hydroxy-Proline (HO-Pro)	100 mM	100	104	102	98	103
	50 mM	100	102	101	102	100
Positive control (PC)		100	36	16	11	11
Negative control (NC)		100	94	93	89	89

Cyanate Scavenging Study

In order to verify the mechanism of the carbamylation protection by the tested compounds, a cyanate level test was performed for 5 mM cyanate in the presence of the above listed compounds and compounds with similar structure, such as diethanolamine, di-peptide HisGly, and tri-peptide GlyGlyGly. Table 3 shows the results of cyanate scavenging study. The protection was calculated based on the percent cyanate remaining from the starting concentration at 2hr and 24 hr time points. All tested compounds showed over 50% cyanate scavenging capability after 24 hr at the concentration of 25 mM or greater, except the diethanolamine. At compound concentrations below 10 mM, the cyanate scavenging potential was not conclusive. The data agreed well with the

results of cyanate carbamylation protection study on RNase A. Based on these results, the

mechanism of the carbamylation protection on RNase A could be attributed to the

cyanate scavenging.

Table 3. Results of cyanate scavenging.

Tested Reagents	Time	CNO⁻ Remaining (%)				
		100 mM	50 mM	25 mM	10 mM	5 mM
1,2-Ethylene diamine	2h	20.0	45.0	NA	67.6	74.6
	24h	0	2.5	16.4	43.8	87.8
Diethanolamine (DEA)	2h	45.4	92.1	87.5	78.2	78.8
	24h	5.5	4.2	59.6	79.7	75.3
Glycylglycine (GlyGly)	2h	23.1	66.4	80.1	79.3	82.7
	24h	0	3.0	11.9	42.4	64.1
L-Histidine (His)	2h	22.9	73.2	91.0	49.5	85.1
	24h	2.0	10.9	32.5	59.6	79.9
Glycinamide (GLA)	2h	29.5	49.5	80.2	70.9	78.7
	24h	2.2	4.4	19.0	0.9	65.7
His-Gly (HisGly)	2h	39.5	52.8	70.9	28.6	81.4
	24h	5.0	20.2	31.5	60.4	81.7
Gly-His (GlyHis)	2h	37.1	49.2	76.7	63.0	86.2
	24h	0.8	6.9	20.9	50.9	77.6
GlyGlyGly (GGG)	2h	42.2	52.6	68.9	50.9	1.2
	24h	1.1	4.6	11.5	81.5	34.1
Hydroxy-proline (HO-Pro)	2h	38.2	40.9	61.2	64.4	61.5
	24h	1.1	8.5	26.6	57.2	72.2

Urea Carbamylation Study

In this experiment, urea in the process buffer was the cause of carbamylation

during protein purification. Cyanate accumulation in the urea buffer is a gradual process.

To demonstrate that the cyanate scavengers is generally applicable to prevent protein

carbamylation during purification, a urea carbamylation study was performed on RNase

A. The urea carbamylation study was set with 1 mg/ml RNase A in 8M urea buffer, pH

7.9 containing different concentration of scavenger reagents over a period of three weeks.

The results of this experiment are summarized in Table 4. The data showed that all tested

scavengers were able to protect RNase A against the carbamylation to some degree. The

trend was the same as observed in cyanate carbamylation study, however, the degrees of

protection observed were consistently lower than those observed from the direct cyanate

carbamylation study. There could be two possible explanations for this discrepancy: the

kinetics might be different in urea, and/or RNase A is unfolded in urea, so more sites are

exposed for carbamylation. The recommended scavenger concentration for preventing

RNase A carbamylation is 25 mM or greater.

Table 4. Results of RNase A urea carbamylation study.

Tested reagents And the concentration		Protection of carbamylation in urea (%)				
		Day 0	Day 3	1 week	2 week	3 week
1,2-Ethylene diamine	50 mM	100	83	62	65	54
	25 mM	100	88	63	61	53
	10 mM	100	83	60	50	37
L-Histidine (His)	50 mM	100	101	98	91	80
	25 mM	100	85	67	41	30
	10 mM	100	84	43	22	16
Glycinamide (GLA)	50 mM	100	83	66	60	51
	25 mM	100	85	64	56	46
	10 mM	100	81	58	39	29
Glycylglycine (GlyGly)	50 mM	100	92	76	64	60
	25 mM	100	88	71	56	50
	10 mM	100	59	62	44	32
His-Gly (HisGly)	50 mM	100	88	67	66	63
	25 mM	100	82	64	52	43
	10 mM	100	82	51	38	27
Gly-His (GlyHis)	50 mM	100	88	69	63	53
	25 mM	100	86	61	49	46
	10 mM	100	84	58	38	27

	50 mM	NA	NA	NA	NA	NA
GlyGlyGly	25 mM	100	85	62	49	43
(GGG)	10 mM	100	85	61	42	28
	50 mM	100	90	69	55	50
Hydroxy-proline	25 mM	100	86	53	47	39
(HO-Pro)	10 mM	100	80	52	31	24
Control	0 mM	100	86	33	13	11

Conclusions

Some compounds, have the potential to prevent protein carbamylation.

Compounds, such as L-Histidine, glycinamide, hydroxyl-proline, di-peptides GlyGly,

GlyHis and HisGly, as well as tri-peptide GlyGlyGly, showed significant protection to

carbamylation of RNase A. The protection of RNase A by the tested compounds is

concentration dependent, with most compounds proficient at 25 mM or greater. RNase A

in that about 20% of the amino acid residues of RNase A are susceptible to

carbamylation, serves as an excellent model protein for the this study. The concentration

of scavenger could vary, depending on the available carbamylation sites of the target

protein. Based on the data collected, the cyanate scavengers tested here can be used in

protein purification processes.

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